(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 November 2001 (22.11.2001)

PCT

(10) International Publication Number
WO 01/88176 A2

(51) International Patent Classification[7]: C12Q

(21) International Application Number: PCT/IL01/00443

(22) International Filing Date: 17 May 2001 (17.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
136232 18 May 2000 (18.05.2000) IL

(71) Applicant *(for all designated States except US)*: **BAR-ILAN UNIVERSITY** [IL/IL]; 52900 Ramat-Gan (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SUNRAY, Merav** [IL/IL]; 7 Hahail Street, 43316 Raanana (IL). **ZURGIL, Naomi** [IL/IL]; 45 Borochov Street, 46240 Hertzlia (IL). **DEUTSCH, Mordechai** [IL/IL]; Moshav Olesh 73, 42855 Lev HaSharon (IL).

(74) Agents: **LUZZATTO, Kfir** et al.; Luzzatto & Luzzatto, P.O. Box 5352, 84152 Beer-Sheva (IL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/88176 A2

(54) Title: MEASUREMENTS OF ENZYMATIC ACTIVITY IN A SINGLE, INDIVIDUAL CELL IN POPULATION

(57) Abstract: A process for measuring enzymatic activity in an identified, isolated, intact, single, viable cell. Each of the viable cells is placed within individual identified locations on a carrier of a cytometer having means to measure enzymatic activity of a single viable cell placed in an identified location. The identified isolated cell is exposed to a substrate of an enzyme to be measured, and the rate of product formed or released following every exposure of the cell to same or different concentrations of the substrate is measured. The isolated cell may be exposed to a sequence of at least two different concentrations of the substrate, and for each exposure the rate of product formed or released, is measured

MEASUREMENTS OF ENZYMATIC ACTIVITY IN A SINGLE, INDIVIDUAL CELL IN POPULATION

Field of the Invention

Enzymes are organic catalysts that cause and direct the numerous chemical reactions that occur in living organisms. Most of chemical changes that occur in living cells are caused and controlled by enzymes. Assessing the enzyme activity in a particular type of cells is therefore one of the principal approaches to the study of what goes on in the same individual living cells.

The present invention provides a new process and methodology for measuring enzymatic activity in intact individual cells. More specifically, it provides the capabilities for high precision enzymatic kinetic measurements of individual cells under repeatable substrate exposure conditions. On-line reagent addition, and controlling other changes in experimental conditions, can be easily accomplished, and the dynamic changes in individual given cells is monitored in real-time. Thus, the process of the invention provides a new valuable tool for assessing enzymatic reaction kinetics, resulting in determination of activity of an individual enzyme as well as of a series of different enzymes, in specific intact cells under defined physiologic conditions.

In a preferred embodiment of present invention, the substrate is either passively or actively enters the cell, once inside, it is processed by the assessed intracellular enzyme to generate detectable product.

In yet another preferred embodiment, the process of present invention is applicable for measuring simultaneously the enzymatic activity in many identified individual cells, within same population.

Since enzymes are ubiquitously involved in cellular function, the monitoring of their reaction kinetics on the level of a single, individual cell may provide valuable information. For example, in some human diseases, especially heritable genetic disorders, there may be a deficiency or even a total absence of one or more enzymes in the tissue. Moreover, measurements of the cellular activity of certain enzymes are important in diagnosing diseases. Most enzymes can be poisoned or inhibited by certain chemical reagents.

Numerous of drugs are designed to inhibit the excessive catalytic activity of specific enzymes in abnormal conditions. Other drugs inhibit certain enzymes in malfunctioning cells. The overall activity of such drugs can only be measured in an intact system of the individual live cell.

An enzymatic activity is usually characterized by two parameters: V_{MAX} – the maximum enzyme production rate (velocity) of a product (P) out of a substrate (S) at a saturation concentration of the latter, and K_M - the Michaelis - Menten

constant, which is reciprocally proportional to the enzyme affinity to the substrate.

The relation between V_{MAX} , K_M, the substrate concentration [S] and the initial velocity *V*, at which *S* converts to P, is given by the Michaelis - Menten equation:

$$V = \frac{[S] \cdot V_{MAX}}{K_M + [S]}.$$

Unfortunately Eq. 1 is accurate only for a homogeneous medium in which the following processes occur:

$[S] + [E] \leftrightarrow [ES]$ and $[ES] \rightarrow [P] + [E]$ where [E] and [*ES*] are the enzyme and the complex enzyme – substrate concentrations, correspondingly.

The determination of K_M and V_{MAX}, utilizing Eq. 1 calls for sequential exposures and repeatable measurements of the same individual cell for various values of [*S*].

Unfortunately this requirement can not be achieved by the common cytometers: The Flow Cytometer (FC) as well as the Laser Scanning Cytometer (LSC). The FC enables the rapid measurement of the fluorescence intensity (FI) of a large cell population. However because each cell in the flow is measured only once, the kinetic curves of the FC

1. Dolbcare F, Fluorescent staining of enzymes for flow cytometry, Methods Cell Biol 33:81-88, 1990

2. Klingel S, Rothe G, Kellerman W, Valet G, Flow cytometric determination of serine proteinase activities in living cells with rhodamine 110 substrates, Methods Cell Biol 41:449-460, 1994

3. Malin-Berdel J, Valet G, Flow cytometric determination of esterase and phosphatase activities and kinetics in hematopoietic cells with fluorogenic substrates, Cytometry 1:222-228, 1980

4. Nooter K, Herweijer H, .Jonker RR, van den Engh GJ, On-line flow cytometry. A versatile method for kinetic measurement, Methods Cell Biol 41:509-526, 1994

5. Turck JJ, Robinson JP, Leucine aminopeptidase activity by flow cytometry, Methods Cell Biol 41:461-468, 1994

6. Watson JV, Dive C, Enzyme kinetics, Methods Cell Biol 41:469-508, 1994]

provide sequential measurements of single cells over time but not of the same single cell. Therefore, investigating different enzyme activities in different cell types or in subcellular areas using the FC gives only an average K_M value for a population of cells or for specific enzymes in a cell-free system.

The LSC measures the fluorescence kinetic of individual cells under specific conditions of low cell density in the selected field and of cell types and dyes which do not suffer from fading, which disrupts the measurement [Watson JV, and Dive C. Enzyme kinetics. Methods Cell Biol (1994) 41:469-508]. The LSC technique cannot ensure the accurate rescanning of the same cell after repeatable staining procedures since the cell may not have preserve its original location. Moreover, the LSC cannot ensure preservation of the cell locations and thus cell identification might be lost during repeatable rinsing and exposure to different substrate concentrations.

In order to provide the capabilities for kinetic measurement of individual cells under repeatable staining conditions, a specially designed cytometer was used. The cytometer (hereinafter referred to as Cellscan Mark S or CS-S) which, one of its versions, was described in the US Patents 4,729,949, 5,272,081, 5,310,674 and 5,506,141 found to be applicable for measuring time resolved kinetics of individual cells during cellular manipulation.

Using the unique application of the CS-S, a new method was developed in which the same cells are sequentially exposed to increasing substrate concentrations. The product formation rate is measured for each cell at every substrate concentration yielding a series of rates for the same individual cell. Using this data, V_{MAX} and apparent K_{MAPP} (app = apparent) values can be calculated for each cell, giving the distribution of K_{MAPP} and V_{MAX} of the measured population

However, it should be emphasized that the process of present invention is not limited to the CS-S cytometer and any cytometer comprising a microscope, light detection means, a carrier to which cells are individually located, is within the scope of the present invention.

Kinetic Analysis:

The kinetic parameters are derived by application of linear and nonlinear modeling. The linear model $y(t)=At+B$ seeks parameters A and B which fit the data to a straight line equation, where $y(t)$ is the measured quantity, t is the time, and A and B are the calculated parameters. The CS-S algorithm uses χ^2 as the criteria for goodness-of-fit.

a. Single Step Cell Staining:

A simplified model for the description of intracellular turnover of fluorogenic substrate is presented in Fig. 1. First, the extracellular substrate $[S]_o$ permeates into the cell, becoming [S]i - the intracellular substrate concentration. Then [S]i is hydrolyzed or cleaved by enzymes to yield the intracellular (for example, fluorescent) product [P]i, which may be released from the cell into the medium and become [P]o .

As was previously shown [Bedner E, Melamed MR, Darzynkiewicz Z, Enzyme kinetic reactions and fluorochrome uptake rates measured in individual cells by laser scanning cytometry, Cytometry 33:1-9, 1998] the kinetics of [P]i can be described, to a good approximation, by the rate equation :

(2) $$\frac{d[P]_i}{dt} = \alpha \cdot [S]_O - \beta \cdot [P]_i$$

Where α and β are the rates constants for the formation and leakage of the intracellular fluorescein. It is important to emphasize that α represents two processes: Permeation of S and its intracellular distribution as well as the enzymatic hydrolysis of $[S]_i$.

When solving Eq. 2, under the initial condition of one step staining, $[P(t=0)]_I = 0$ it is easily shown that

(3)

$$P(t) = \frac{\alpha}{\beta}[S]_O(1 - e^{-\beta \cdot t})$$

b. Sequential staining:

Another aspect of present invention relates to sequential exposures of the same individual cells to different substrate concentrations. This differs from the above case by the fact that at the starting time point of staining, with a given solution, cells are already being stained to a level of:

(4) $$\left[P(\tau)\right]_i = \frac{\alpha}{\beta} M[S](1-e^{-\beta\tau})$$

τ stands for the time point of terminating the staining with a given substrate concentration, say M times $[S]$ ($M[S]$),and initiation of staining with different substrate concentration, say $N[S]$.

Now, it is possible to solve Eq.2 under the initial conditions presented by Eq. 4. By separation of variables and integration over $[P]_i$ between the concentration limits $[P(\tau)]_i$ and $[P(t)]_i$; and integration over time between the time points 0 (when staining solutions are being replaced) and t, one gets:

(5)

$$\int_{[F(\tau)]_I}^{[F(t)]_I} \frac{d[F]_I}{[F]_I - \frac{\alpha}{\beta}N[S]} = \int_0^t -\beta dt \Rightarrow \ell n\left(\frac{[F(t)]_I - \frac{\alpha}{\beta}N[S]}{[F(\tau)]_I - \frac{\alpha}{\beta}N[S]}\right) = -\beta \cdot t$$

Converting the logarithmic expression into exponential one and introducing $[F(\tau)]_I$ of Eq.4 into Eq.5 yields:

(6)

$$\left[F(t)\right]_I = \frac{\alpha}{\beta} M\left[S\right](1-e^{-\beta\tau})e^{-\beta t} + \frac{\alpha}{\beta} N[S](1-e^{-\beta t})$$

When single step staining is performed (starting of unstained cell, M=0), only the last term of Eq. 6 remains, which is consistent with Eq. 3.

As long as the expression $\exp(-\beta t) \cong 1-\beta t$ holds for the duration of the observation interval of the individual cells in given conditions, regardless of their staining history, each of the exponential terms in Eq. 6 can be replaced, without losing accuracy, by its first two terms of the power series. Hence, Eq. 6 may be linearly approximated to give:

(7)

$$[F(t)]_I = \begin{cases} 0 < t < \tau \\ t > \tau \end{cases} \rightarrow \begin{matrix} \alpha[S]Mt \\ \alpha[S]\cdot(M\tau + Nt) \end{matrix} \rightarrow \begin{matrix} \frac{d[F(t)]_I}{dt} = \alpha[S]M \\ \frac{d[F(t)]_I}{dt} = \alpha[S]N \end{matrix} \}$$

Eq. 7 should be interpreted as follows: for $0< t<\tau$, staining proceeds according to $[P(t)]_i = \alpha[S]Mt$. After replacing the staining solution M by N at time $t=\tau$, the staining due to $M[S]$ remain constant $[P(\tau)]_I = \alpha[S]M\tau$, While that due to N increases at a rate of $\alpha[S]N$, namely solely depending on the concentration in use. Simulations of several practical staining protocols, based on Eq.7, are graphically presented in Fig. 2 and briefly described in the following:

a) Rinsing the cells with a staining solution $[N]$ that maintains $[N] = [M]$, results in a staining curve $[P(t)]_I=\alpha[S]N[\tau+t]$. At the observation time $\tau+t$ $[P]_i$ had a production rate of $\alpha[S]N$, the same rate as that of $\alpha[S]M$ prior to $\tau+t$ (Fig. 2a).

b) Rinsing the cells with PBS alone washed away $[M]$ residues leaving the staining solution at a concentration $[M] = 0$. This action halted any further production $[P]_I$ (since $\alpha[S]N = 0$ at the time of application ι) hence $[P]_i$ line remained parallel to the time axis for the duration of the observation t. (Fig. 2b).

c) In a similar way, the cells were rinsed with a staining solution $[N] \neq [M]$ that washed away $[M]$ and left the staining solution at a concentration $[N]$. The production rate of $[P]_i$, as expected, changed to $\alpha N [S]$ for the observation duration t. (Fig. 2c)

d) The last experiment, was a combination of b) and c) in succession. First the cells were rinsed at time t_1 with PBS and that halted the production of F_I. The next stage was to rinse with a staining solution $[N] \neq [M]$ replacing the PBS with a solution of concentration $[N]$. The production rate then changed to $\alpha[S]N$ for the for the observation duration t. (Fig. 2d).

Finally, the determination of Δt, the overall sequential staining experiment procedure time duration, was restricted to follow the present CS-S Standard deviation in performing individual cell FI measurements, which is < 2%.

In order not to exceed this value when linearly approximating the exponential terms, a Δt value which keeps the ratio

$\exp(-\beta\Delta t)/(1-\beta\Delta t) \cong 2\%$ is sought. Hence, introducing $\beta \cong 10^{-4}$ sec^{-1}, which is the outcome of many hundreds of independent experiments (data not shown), yields $\Delta t \cong 103$ sec.

Summary of the Invention

It is an object of present invention to provide a process for measurement and characterization of intra- and extra-cellular enzymatic activity taking place in the same identified individual cell, in a population of cells, following its incubation with different concentrations of a substrate. The substrate should be a substance that yields a product that is detectable by physical means, such as changes in fluorescence intensity, color intensity, radioactive radiation, etc.

It is a further object of present invention to establish a new method for the determination of K_{MAPP} and V_{MAX} values for enzymatic reactions carried out inside an identified individual cell. It is an additional object of the present invention to determine kinetic values of extracellular enzymes, released from an individual cell. It is yet an additional object of present invention to provide a tool for measuring differences in kinetic enzymatic activity in the individual cell following various treatments of same cell with biologically active materials.

A further object of present invention is to provide a process for measuring simultaneously the enzymatic activity in many identified individual cells, within same population.

Brief Description of the Drawings

Fig. 1: A model of intracellular conversion of a substrate to a product. $[S]_0$, $[S]_i$ are the extracellular and intracellular substrate concentrations and $[P]_0$, $[P]_I$ are the extracellular and intracellular product concentrations. [E] and [ES] are the enzyme and enzyme substrate complex concentrations. k_1 is the rate constant for formation of the complex [ES], k_{-1} is the rate constant for the reversed reaction and k_2 is the rate constant for product formation.

Fig. 2: Simulation of an individual cell sequential FI time dependency following several exposure procedures to substrate concentration. M = multiplication coefficients of initial substrate concentration.

R = rinsing at a given time point. Panels: a - rinsing with the same concentration yields identical slopes. b - sequential rinsing with (yield identical slopes as in panel a) and without (zero slopes) substrate. c - sequential rinsing with increasing substrate concentrations. d - sequential rinsing with increasing substrate concentrations while in between rinsing without substrate.

Fig. 3: Experimental results of individual cells sequential staining procedure. The numbers in the boxes are the slopes of FI(t) (initial velocities), given in arbitrary intensity units per second. The experiment follows the simulation shown in Fig. 2.

Fig. 4: Complete sequential staining procedure of numerous cells. Each of the

four clusters contains 13 lines. Each line defined by six FI measurements taken in six different time points for the same individual cell when exposed to the relevant substrate concentration. R_1 to R_4 - the space between clusters stands for replacement duration of the staining solutions (0.6, 1.2, 2.4 and 3.6.μM). The solid line in each of the four clusters is sketched for clarification purposes. It indicates the increasing slopes of one chosen set of sequential exposure of one individual cell.

Fig. 5: Individual K_{MAPP} and V_{MAX} for two representative cells and their Pearson correlation coefficient (R^2).

Fig. 6: The distribution of individual K_{MAPP} (6A) and V_{MAX} (6B) for cells that were incubated with (—) and without (- - -) PHA.

Fig. 7: Rate of change of FI before and after exposure of an individual cell to hydrogen peroxide (H_2O_2) compared with control. The ratio pre to post treatment slopes in control cells is double that of cells exposed to H_2O_2 (treated).

The following examples are provided merely to illustrate the invention and are not intended to limit the scope of the invention in any manner.

Examples

Example 1. Measuring intracellular nonspecific esterase activity in a single lymphocyte using fluorescein-diacetate (FDA) as the substrate.

Materials and Methods

Phytohemagglutinin PHA (*HA15, Murex Biotech*) was reconstituted in 5 ml of double-distilled water and further diluted ten times. For stimulation, 10µl of this solution was added to a 90 µl cell suspension ($7x10^6$ cells/ml).

The culture medium consisted of RPMI-1640 (Biological Industries), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biological Industries), 2mM L-glutamine, 10 mM Hepes buffer solution, 1mM sodium pyruvate, 50 U/ml penicillin and 50Units/ml streptomycin.

A staining solution of 3.6 µM FDA (Riedel-de Haen Ag. Seelze-Hanover) in Dulbecco Phosphate Buffered Saline (PBS, Biological Industries) was prepared as follows: 50 mg of FDA was dissolved in 5 ml of DMSO (Sigma). 7.5 µl of this solution was added to 50 ml PBS. For 0.6, 1.2 and 2.4µM the solution was further diluted in PBS.

Preparation of Peripheral Blood Mononuclear Cells (PBMC):

30 Heparinized blood (30 ml), was taken from healthy, normal volunteers. The procedure for separating the PBMC has been described in detail, elsewhere [Sunray M, Deutsch M, Kaufman M, Tirosh R, Weinreb A, and Rachmani H. Cell Activation influences cell staining kinetics, Spectrochimica Acta A (1997) 53:1645-1653]. Shortly after removing the iron absorbing cells, the remaining cells are layered on a two-layer (100% and 80%) cell density gradient (Ficoll Paque, Pharmacia 1.077 g/ml) and centrifuged. The cells accumulated at the

interface between the two Ficoll layers, were collected and kept at 37° C in 5 ml of enriched culture medium overnight. The next day the PBMC were washed and resuspended in PBS at a final concentration of $7 \cdot 10^6$ cells/ml. More than 70% of the cells were defined as T lymphocytes and viability, which was determined using eosin, was always higher than 90%.

Activation of PBMC by PHA:

Freshly prepared PBMC ($7 \cdot 10^6$ cells/ml) were incubated at 37°C, 5% CO2 with 5 μgr/ml PHA for 30 minutes. PBMC controls were incubated without PHA under identical conditions.

The CS-S Apparatus

The multiparametric, computerized, discrete cytometer CS-S used in performing this example was described in detail in the above specified US Patents. Its central feature is a cell carrier (CC) incorporating a 100 × 100 dimensional array having a conical cross-section with an upper opening of ~7μm and lower opening of ~4μm, each approximately 20μm apart, in which individual cells are trapped. The cell carrier is mounted on a computer-controlled stage that enables repeated multi-scanning of the same cells.

Cells were irradiated with 1-10 μW of 442 nm light from a He-Cd laser. Under the staining conditions used here, the scanning time for obtaining a count of 10,000 photons in order to have statistical photonic error of ~1% from each, dye-loaded cell varied from 0.001 sec to approximately 0.5 sec.

The acquired data, including cell position, measurement duration for each cell, absolute time, intensity at two different wavelengths, computed fluorescence polarization values and test set-up information, are displayed on the screen, on-line, graphically and numerically, and stored in the memory. Software enables the determination of the range and other statistical characteristics of all parameters, for either the entire cell population, or an operator-selected sub-population, or an individual cell, before, or during the scan.

Cell Loading

Loading the cells in wells traps on the Cell Carrier (CC) was carried out, as described in Deutsch M, and Weinreb A., Apparatus for High Precision Repetitive Sequential Optical Measurement of Living Cells, Cytometry (1994) 16: 214-226. An aliquot of 80 μl of unstained cell suspension (7×106 cells/ml) was loaded on the CC. Initial scanning was then performed in order to detect individual cell background scattering and auto-fluorescence. This undesired signal is recorded per measurement location and subtracted from the total emission signal (after exposure) in order to obtain the correct fluorescence signal.

Cell Staining and Kinetic Measurement:

For fluorescence intensity FI(t) measurements, trapped cells on the CC were sequentially exposed to increasing concentrations of FDA in PBS staining solutions.

Following background measurement, the volume of PBS, which covers the cells, was pumped out and the following procedure was carried out:

At time point zero, 40 µl of the lowest substrate concentration solution was applied on top of the trapped cells and a pre-chosen cell field was sequentially scanned 6 times. This yielded 6 accurately timed FI data points per each individual cell at a given dye concentration. FI is usually measured utilizing epi-fluoescence optical arrangement which permits the differentiation between the excitation energy and the emitted fluorescence energy to be detected by photomultipliers, CCD detectors etc.

The above procedure is repeated for each different substrate solution used in the experiment.

This yielded six FI data points for each individual cell, per substrate concentration, from which V was extracted and the individual cell K_{MAPP} and V_{MAX} values were calculated. The dead time, i.e., the elapsed time from the addition of a staining solution to the beginning of the measurement, which is monitored by the computer, is about 7-15 sec.

Results

Repeatability runs:

The experimental arrangement of the new process calls for high-level performance in terms of repeatability and accuracy in periodical measurements of individual cells.

The CS-S capability was displayed by performing sequential measurements of FI

and FP on 5 min 1.2μM FDA stained trapped cells, following their PBS rinsing out of excess substrate solution and possible extra-cellular P_I (at this stage, constancy of FI is expected due to staining termination and negligibility of P_I leakage).

The individual cell coefficient of variance (CV) obtained in more then 10 successive measurement scans of a 10 × 10 cell field, never exceeded 2% for FI. Fading was not noticeable.

Accuracy Runs:

Accurate intensity measurement capabilities and specific monitoring of alterations in F_I production rate are mandatory for the present process. This was first serially examined by measuring FI of the CC loaded with cell-free fluorescein solutions at concentrations of 0.6, 1.2 and 2.4μM, five times each, while rinsing with PBS between concentrations.

The ratios, $FI([s]_i)/FI([s]_j)$, between the measured FI, for different [s] and fluorescein concentrations, were found to be in correlation to the ratios of FDA substrate concentrations ($[s]_I/[s]_J$) and free fluorescein concentrations ($[F]_i/[F]_j$), (see Table 1).

The correlation between the substrate concentration (FDA concentration) and the measured staining rates by intracellular fluorescein was established for cells. First, each CC was loaded with unstained (BPS free of substrate) cells and

stained with one chosen substrate concentrations (in order to avoid possible influences of additive staining when sequential exposure is performed).

Second the sequential staining manipulation was examined. As can be seen in the third and forth column of Table 1, there was good correlation between the increasing staining rates (which means increasing rate of product formed) and the increasing substrate concentrations in both cases.

Next, using the sequential staining manipulation [adding in sequence of different concentrations of substrate and measuring the production rate of F in between additions, by monitoring FI(t)] with cells, it was verified the theory described in equation 7 specifically for the four cases that are detailed above and are presented as simulations in Fig. 2.

First, cells were loaded on the CC and stained with FDA. Re-washing the cells after every five or six scans with the same FDA concentration gave similar slopes after every wash as can be seen for FDA at 1.5μM in Fig. 3a.

Rinsing (R) the cells with FDA and with PBS (no FDA, N=0, equation 7) alternately gave similar slopes when FDA was present and almost zero slope when FDA was absent, as shown in Fig 3b.

The level of FI at the beginning of the last rinse was higher than the level at the end of the rinse with PBS though the slopes (velocities which are the magnitude used for K_{MAPP} determination) were identical. This difference is probably due to technical reasons such as a slight change in the focus while manipulating FDA

concentration or by laser beam geometrical instability etc. In Fig. 3c the cells were rinsed with increasing FDA concentration of 0.6, 1.2, 2.4 and 3.6μM.

In Fig. 3d, the cells were rinsed with FDA at concentration of 0.6, 1.2, 2.4 μM and in between with PBS without FDA. The PBS gave almost zero slopes (no production of FI) while the increasing FI slopes were in good correlation with the increasing FDA concentration. Generally, as can be seen from figures 2 and 3, there was good correlation between the theoretical simulation and results of the experiments.

Table 1

Substrate concentrations [s] ratio (μM) (a)		Ratios of FI of various concentrations of fluorescein solutions (b)	FI rate ratios of cells parallely exposed to different FDA concentrations Each on different cell Carriers (c)	FI rate ratios of the same trapped cells sequentially exposed to different FDA concentrations (d)
2.0	1.2/0.6	2.1	1.9	2.1
4.0	2.4/0.6	4.0	4.4	3.6
6.0	3.6/0.6	------	6.2	5.6

Table 1: Ratios of substrate concentrations (a); of fluorescein solutions FI (b); and of intracellular fluorescein production rates of trapped cells: (c) parallely exposed on different CC each to different FDA concentrations and (d) sequentially exposed to different FDA concentrations on the same CC.

Determination of Individual K_{MAPP} and V_{MAX} Values:

Determination of K_{MAPP} and V_{MAX} was carried out by utilizing the reciprocal of Eq. 8 (Lineweaver - Burk plot)

$$\frac{1}{V}=\frac{K_M}{V_{MAX}}\frac{1}{[S]}+\frac{1}{V_{MAX}} \qquad (8)$$

Thus, the use of two substrate concentrations should be, in principal, enough for the extraction of K_M and V_{MAX} . Minimization of possible experimental errors, while restricted by the linear range of time duration, $\Delta t \cong 10^3$ sec, led to the choice of 4 FDA concentrations: 0.6, 1.2, 2.4 and 3.6 μM. Practically, trapped cells on the cell carrier were sequentially exposed to the four FDA concentrations staining solutions and scanned for determination of released fluorescein six times per the same FDA concentration. A representative chart of a complete measurement procedure made on 50 cells is shown in Fig. 4.

A plot of Eq. 8 for two cells out of the measured population of Fig. 4 is presented in Fig. 5.

Example 2.

Utilization of Individual K_{MAPP} Measurements

The Influence of Mitogenic Stimulation upon K_{MAPP} and V_{MAX}:

The activation of lymphocytes is a critical stage in most immune responses and allows these cells to exert their specific functional capabilities. During activation, the resting lymphocytes undergo complex changes resulting in cell differentiation and proliferation. Lymphocytes activation is triggered by multiple interactions that occur at the cell surface, which initiate intracellular biochemical events within the cell that culminate in cellular response.

One of the experimental models used to study lymphocytes activation is lectins, plant derived proteins (including phytohemagglutinin PHA), that bind carbohydrate groups at the cell surface and stimulate relevant receptors involved in physiologic lymphocyte activation. Many pharmacological agents mimic or inhibit some of the intracellular events associated with T cell activation. An example is described herein for individual K_{MAPP} measurement following lymphocyte activation.

The sequential FDA hydrolysis experiment was executed following incubation of cells with and without phytohemagglutinin PHA. The distribution of individual K_{MAPP} and V_{MAX} values for both cases are presented in Fig. 6a and 6b, respectively. The average K_{MAPP} and V_{MAX} were found to be 4.88 μM and 1.50 μM and 695 (intensity/sec) and 652 (intensity/sec), indicating a decrease of 69% in K_{MAPP} and 6% in V_{MAX} values for PHA compared to the control. Both distributions indicated cell heterogeneity having a CV of about 70%.

For comparison purposes, at the average level, the FC (Beckton-Dickinson FACSCalibur) was used to determine K_{MAPP} and V_{MAX} value averages taken over the cell population following the protocol suggested by Watson, J.V and Dive, C., Enzyme Kinetics. Methods Cell Biol (1994) 41: 469-508. Four means of intracellular fluorescence intensities (IFI) were calculated from data accumulated along four time gates of 25 second each and 30 seconds apart, from which V_0 was extracted. This process was sequentially performed on five different aliquots of cells (50 µl, at a concentration of $6x10^6$ cells/ml) each exposed to different FDA concentrations (0.3, 0.6, 1.2, 1.8 and 2.4 µM). Introducing these average V_0 in values Eq. 8 yielded population average K_{MAPP} and V_{MAX} of 2.16µM and 4.32µM and 6.6 and 5.83 in cells incubated with and without PHA. It should be noted that while K_{MAPP} is an intrinsic value, V_{MAX} depends on the optoelelctronic arrangement under use. Thus, obviously, at the population level, measurements carried out both on FC and average calculated from individual cell K_{MAPP} measurement data yield similar K_{MAPP} values, indicating the validity of the invented methodology.

Example 3.

Using basically the same procedure, it is possible to determine the following enzymes activity in the single, individual cell:

1. Proteases and Peptidases

Peptidases and proteases play essential roles in protein activation, cell regulation and signaling, as well as in the generation of amino acids for protein synthesis or utilization in other metabolic pathways. Typical peptidase substrates are short peptides conjugated to fluorophores (like 7-Amino-4-methylcoumarin (AMC) or

Rhodamine 110). In the presence of the enzyme, the fluorogenic part is released, and may be easily determined by fluorescence measurements. One example of peptidase is the cystein protease- Caspase which play a pivotal role in programmed cell death.

AMC- and R110-labeled peptidase substrates, permit the detection of apoptosis by assaying for increases in caspase-3 and caspase-3–like protease activities. The activation of caspase-3 (CPP32/apopain), which has a substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves a number of different proteins, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C and actin, is important for the initiation of apoptosis. Both substrates can be used to continuously measure the activity of caspase-3.

2. Peroxidases

Reactive oxygen species, including singlet oxygen, superoxide, hydroxyl radical and various peroxides (ROOR') and hydroperoxides (ROOH).are produced during a number of physiological processes. Activated oxygen species react with a large variety of easily oxidizable cellular components, including NADH, NADPH, dopa, ascorbic acid, histidine, tryptophan, tyrosine, cysteine, glutathione, proteins and nucleic acids. Reactive oxygen species can also oxidize cholesterol and unsaturated fatty acids, causing membrane lipid peroxidation. The importance of the nitric oxide radical enzyme producer and other reactive oxygen species as biological messengers has been increasingly recognized during the last several years. Assaying of oxidative activity in live cells can be done by using Leuco Dyes. Fluorescein, rhodamine and various other dyes can be chemically reduced

to colorless, non-fluorescent leuco dyes. These "dihydro" derivatives are readily oxidized back to the parent dye by some reactive oxygen species and thus can serve as fluorogenic probes for detecting oxidative activity in cells. Dihydroethidium, dichlorodihydrofluorescein (H2DCF) and dihydrorhodamine 123 react with intracellular hydrogen peroxide — a reaction mediated by peroxidase, cytochrome C or Fe^{2+}. The leuco dyes also serve as fluorogenic substrates for peroxidase enzymes.

3. Glucose Oxidase

The enzyme glucose oxidase is widely used for glucose determination. Glucose oxidase reacts with glucose to form gluconolactone and H_2O_2. The H_2O_2 is then detected using fluorescent probe as described above.

4. Carbonic Anhydrase

Carbonic anhydrase catalyzes the reversible hydration of CO_2 to carbonic acid. Acetazolamide has been shown to bind carbonic anhydrases in a wide variety of eukaryotic cells. Fluorescent-labeled derivative of acetazolamide is used for studying carbonic anhydrase activity in live cells.

As was described hereinabove, a major embodiment of present invention involves the measurement in individual cells of K_{MAPP} and V_{MAX} values of particular cellular enzymes. This is a rather important assay relating to drug activity within a single intact cell.

In general, pre drug-treated cells are exposed to at least 2 different substrate concentrations in order to determine the enzymatic K_{MAPP} and V_{MAX} values. The

same cells are then exposed to the investigated drug (or any other biologically active material, such as ,inducer, inhibitor,etc.), during a selected period of time. Finally, the cells are again exposed either to the same 2 substrate concentrations or another 2 or more substrate concentrations, and the K_{MAPP} and V_{MAX} values of the drug-treated cells, is determined.

In the following, an example is given in order to demonstrate this principle. Peripheral blood lymphocytes were loaded on a CC, and exposed to FDA, after which individual FI(t) was measured. The same trapped cells, on the same CC were then rinsed (R) twice with fresh buffer and incubated at 37°C in the presence of hydrogen peroxide (an apoptotic inducer). At the end of incubation, the same cells were again exposed to the same FDA concentration and FI(t) measurements were again performed.

Despite the fact that such an experimental procedure is self consistent (since it has its own control on an individual cell basis, namely control measurements of K_{MAPP} and V_{MAX} of cells, prior to their incubation with the drug), an additional experiment was carried out as a second external control, but this time cells were incubated without the drug.

FI(t) of two representative cells, measured prior to and after incubation with (treated) and without (control) hydrogen peroxide (the drug) are shown in Fig. 7. Since cells are in general heterogeneous, one would expect a distribution of FI(t) rates (slopes) in the same experiment. This is why the initial slopes (V_0) of the

two curves in Fig. 7 are not identical. Thus, in such an experimental procedure, the determining parameter is the ratio between the initial and the final slopes, namely, the ratios between FI(t) slopes prior to and after incubation (with and without drugs), as well as ratios of individual K_{MAPP} and V_{MAX} prior to and after incubation.

Calculation of both slope ratios shown in Fig. 7 indicates that exposure of lymphocytes to mild oxidative stress resulted in a lower rate of the second staining reaction, in comparison to control. The ratio between the first and the second reactions reflected the apoptotic activity of the inducer Moreover, it can provide an idea regarding apoptotic resistance of specific individual cells.

CLAIMS

1. A process for measuring enzymatic activity in an identified, isolated, intact, single, viable cell, comprising the steps:

(a) placing each of the viable cells within individual identified locations on a carrier of a cytometer having means to measure enzymatic activity of a single viable cell placed in an identified location,

(b) exposing the identified isolated cell to a substrate of an enzyme to be measured, and

(c) measuring the rate of product formed or released following every exposure of the cell to same or different concentrations of the substrate.

2. A process according to claim 1, wherein the isolated cell is exposed to a sequence of at least two different concentrations of the substrate and for each exposure the rate of product formed or released, is measured.

3. A process according to claim 2 for measuring the kinetic of a particular enzyme, wherein initial rate production (V_O-velocities) are measured from which V_{MAX} and K_M are calculated.

4. A process according to claim 1, wherein activities of several different enzymes are measured in the same isolated cell in a population.

5. A process according to claim 1, wherein activity of a particular enzyme is measured before and after the treatment of said isolated cell with a biologically active material.

6. A process according to claim 5, wherein the biologically active material is a drug.

7. A process according to claim 5, wherein the biologically active material is an inhibitor of any of the treated cell's functions.

8. A process according to claim 5, wherein the biologically active material stimulates, induces or promotes a particular function or property of the treated cell.

9. A process according to claim 5, wherein the production rates (V_O) are measured and V_{MAX} and K_M are calculated before and after cell treatments.

10. A process according to claim 1, wherein the substrate consists of a known fluorescent substance that as a result of enzymatic activity is converted into a measurable fluorescentic product.

11. A process according to claim 10, wherein the substrate is fluorescein-diacetate (FDA).

12. A process according to claim 1, wherein the measured activity is of an intra-cellular enzyme.

13. A process according to claim 12, wherein the intra-cellular enzyme is selected from the group comprising esterase, protease, peptidase, peroxidase, glucose oxidase and carbonic anhydrase.

14. A process according to claim 1, wherein the measured activity is of an extra-cellular enzyme.

15. A process according to claim 1 wherein the isolated single cell is a lymphocyte.

16. A process according to claim 1, wherein the isolated single cell is a lymphocyte, the enzyme is an esterase and the substrate is fluorescein-diacetate.

17. A process according to claim 1, wherein the substrate is color-less and the product formed or released is colored.

18. A process according to claim 1, wherein the substrate is colored and the product formed or released is color-less.

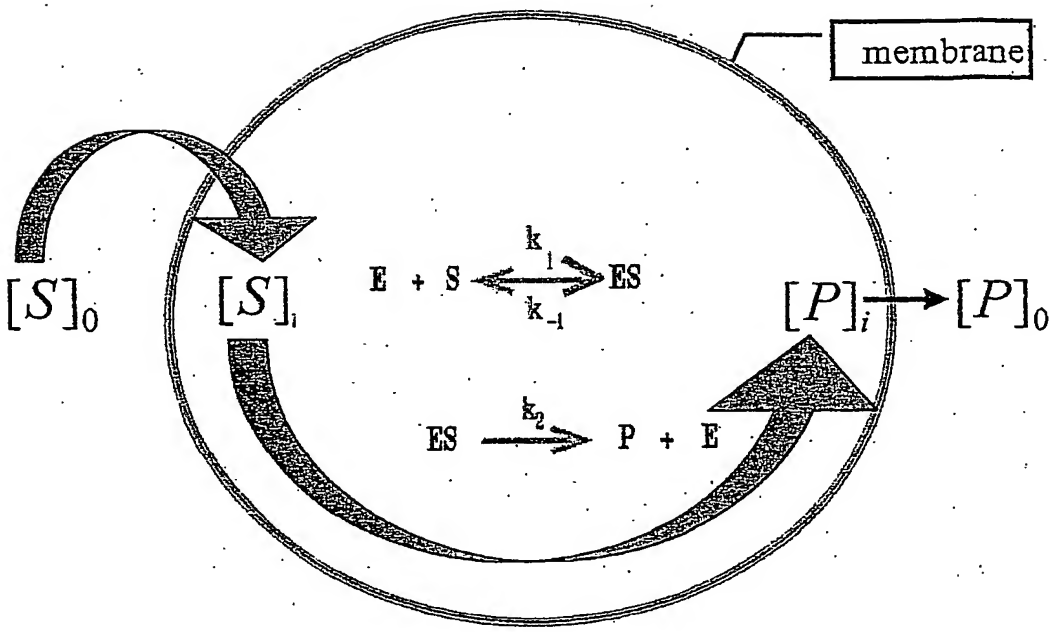


Fig. 1

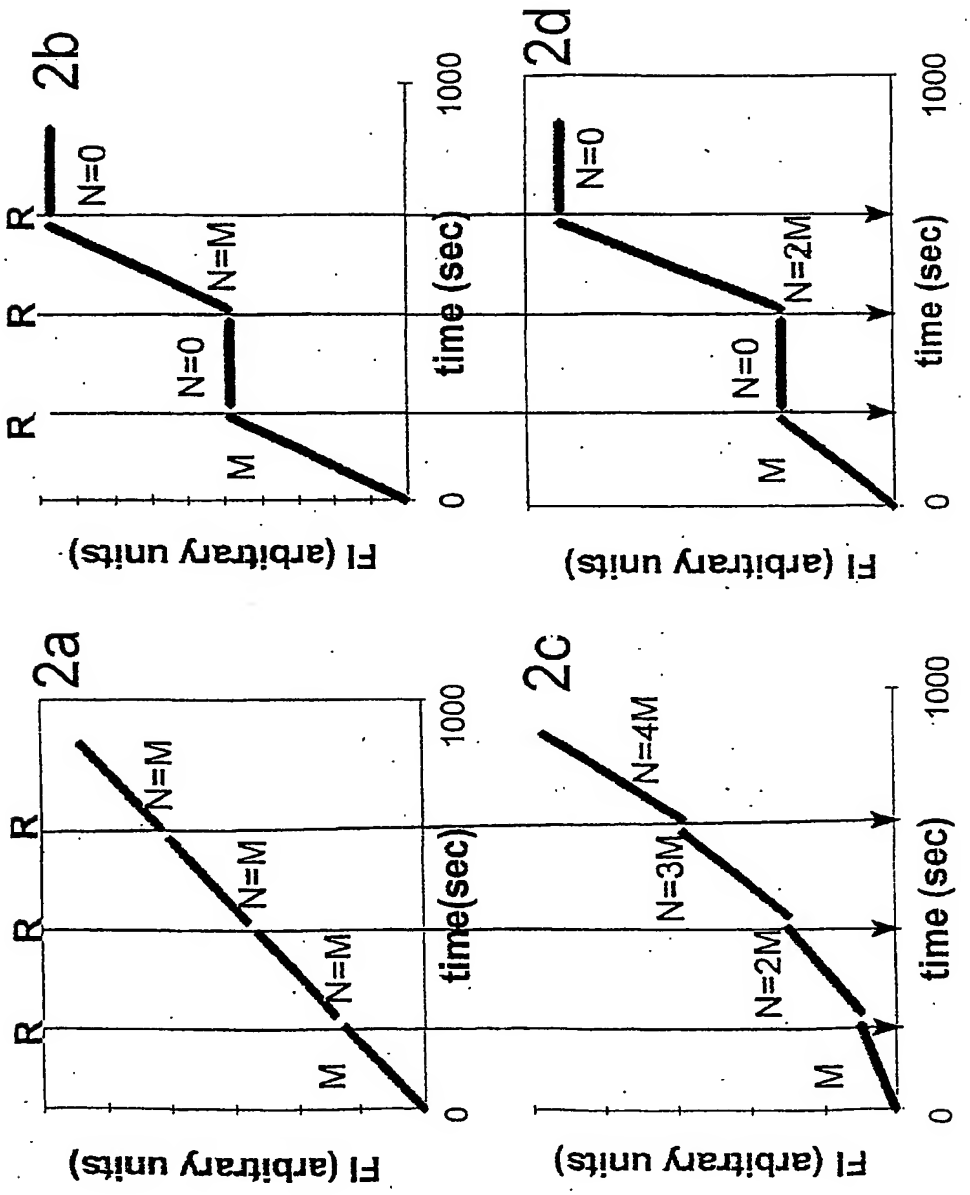

2b
2d
2a
2c
R
N=0
N=M
N=2M
N=3M
N=4M
M
time (sec)
time(sec)
0
1000
FI (arbitrary units)

Fig. 2

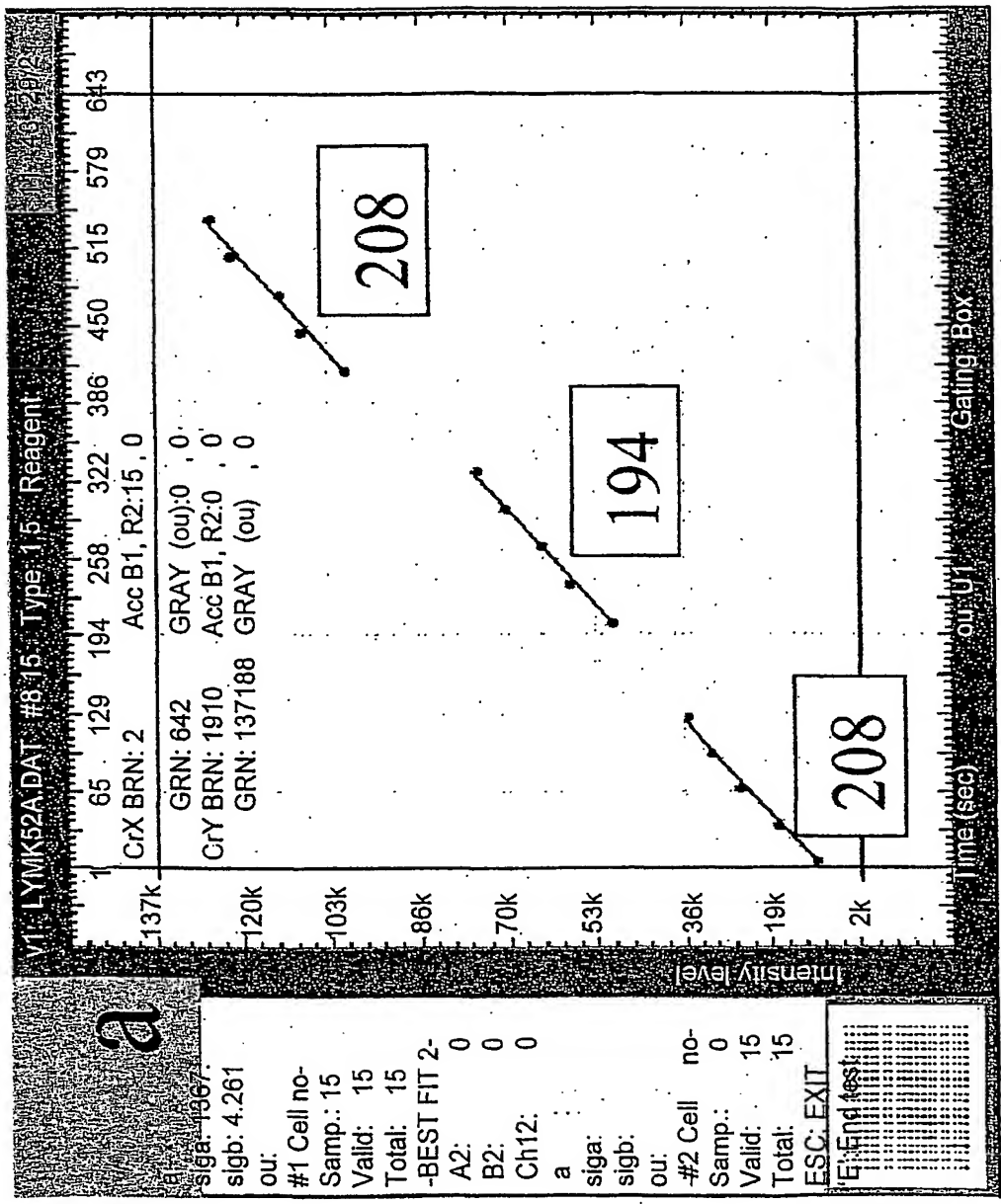

a
V1: LYMK52A.DAT #8 15 Type: 1 5 Reagent
1 65 129 194 258 322 386 450 515 579 643
CrX BRN: 2 Acc B1, R2:15 , 0
GRN: 642 GRAY (ou):0 , 0
CrY BRN: 1910 Acc B1, R2:0 , 0
GRN: 137188 GRAY (ou) , 0
137k
120k
103k
86k
70k
53k
36k
19k
2k
Intensity level
Time (sec)
208
194
208
Gating Box
siga: 1367.
sigb: 4.261
ou:
#1 Cell no-
Samp.: 15
Valid: 15
Total: 15
-BEST FIT 2-
A2: 0
B2: 0
Ch12: 0
a
siga:
sigb:
ou:
-#2 Cell no-
Samp.: 0
Valid: 15
Total: 15
ESC: EXIT
E: End test

Fig. 3A

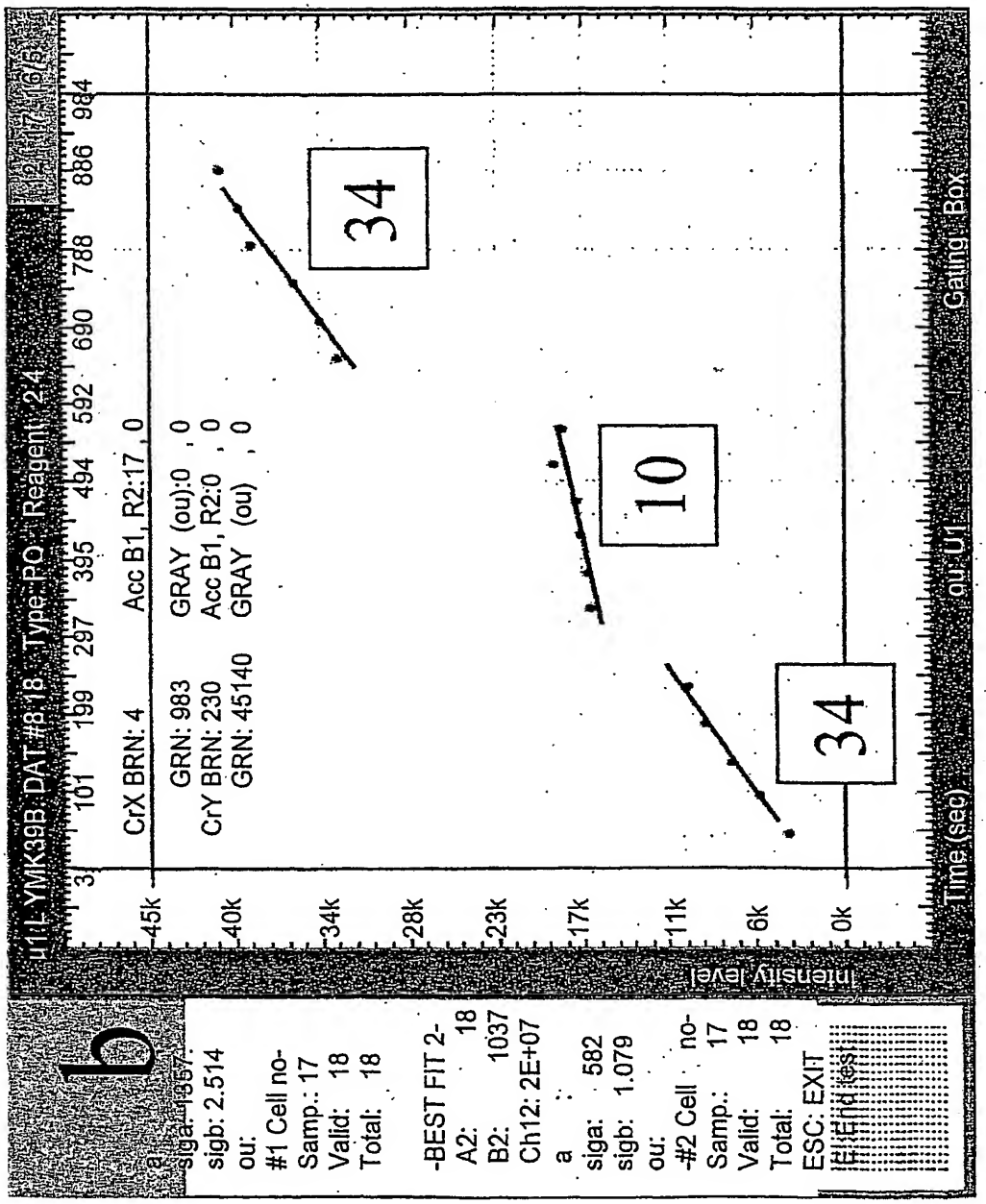

B
U1:L YMK39B: DAT #8:18 Type:RO Reagent: 2:4
3 101 199 297 395 494 592 690 788 886 984
CrX BRN: 4 Acc B1, R2:17 ,0
GRN: 983 GRAY (ou):0 ,0
CrY BRN: 230 Acc B1, R2:0 ,0
GRN: 45140 GRAY (ou) ,0
34
10
34
45k
40k
34k
28k
23k
17k
11k
6k
0k
Intensity level
Time (sec)
ou: 0
Gating: Box
siga: 1357
sigb: 2.514
ou:
#1 Cell no-
Samp.: 17
Valid: 18
Total: 18
-BEST FIT 2-
A2: 18
B2: 1037
Ch12: 2E+07
a :
siga: 582
sigb: 1.079
ou:
#2 Cell no-
Samp.: 17
Valid: 18
Total: 18
ESC: EXIT

Fig. 3B

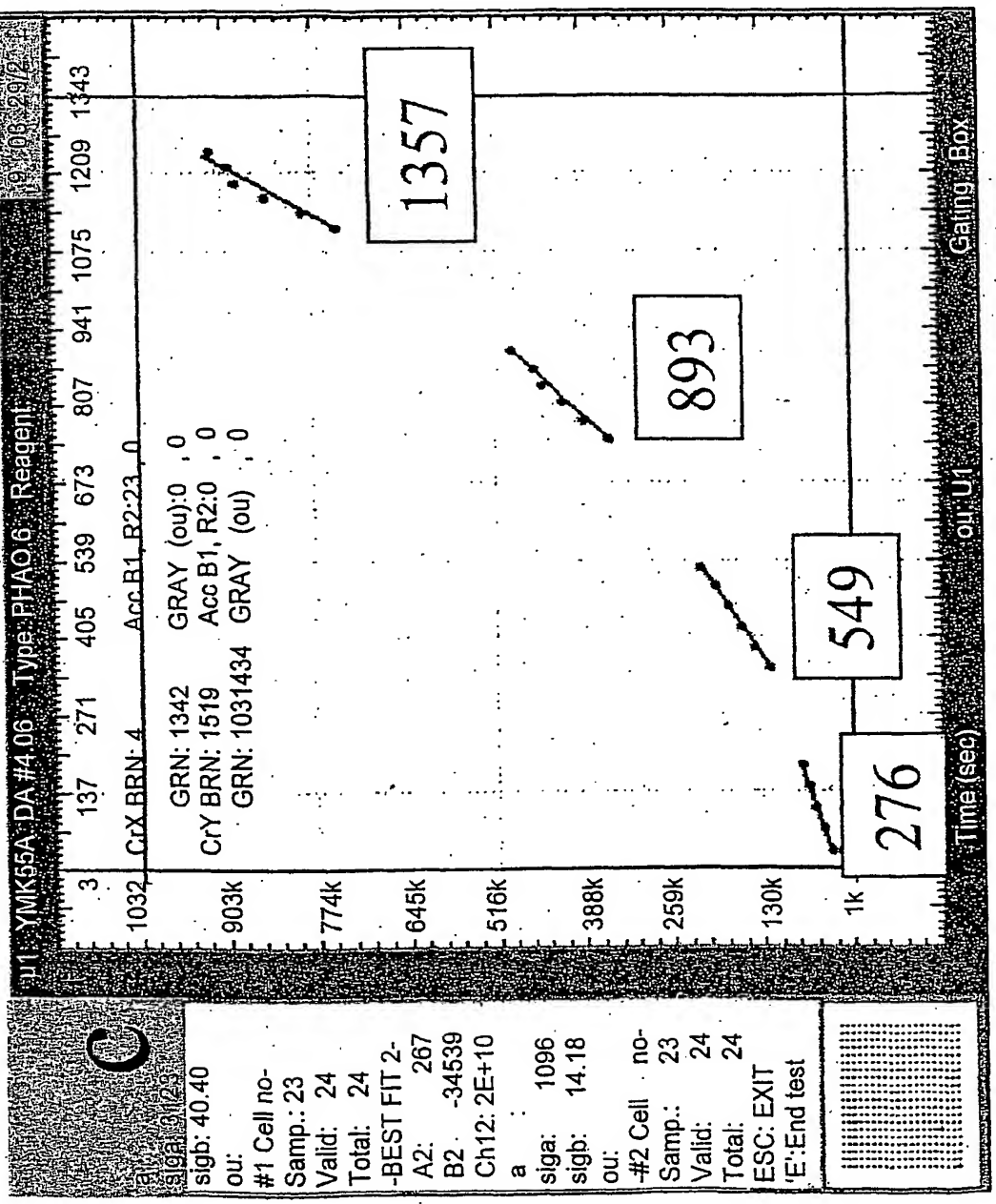

P1: YMK55A DA #4.06 Type: PHAO.6 Reagent:
3 137 271 405 539 673 807 941 1075 1209 1343
1032
903k
774k
645k
516k
388k
259k
130k
1k
CrX BRN: 4 Acc B1, R2:23 , 0
GRN: 1342 GRAY (ou):0 , 0
CrY BRN: 1519 Acc B1, R2:0 , 0
GRN: 1031434 GRAY (ou) , 0
1357
893
549
276
Time (sec)
ou: U1
Gating Box
siga: 31123
sigb: 40.40
ou:
#1 Cell no-
Samp.: 23
Valid: 24
Total: 24
-BEST FIT 2-
A2: 267
B2 -34539
Ch12: 2E+10
a
siga: 1096
sigb: 14.18
ou:
#2 Cell no-
Samp.: 23
Valid: 24
Total: 24
ESC: EXIT
'E':End test

Fig. 3C

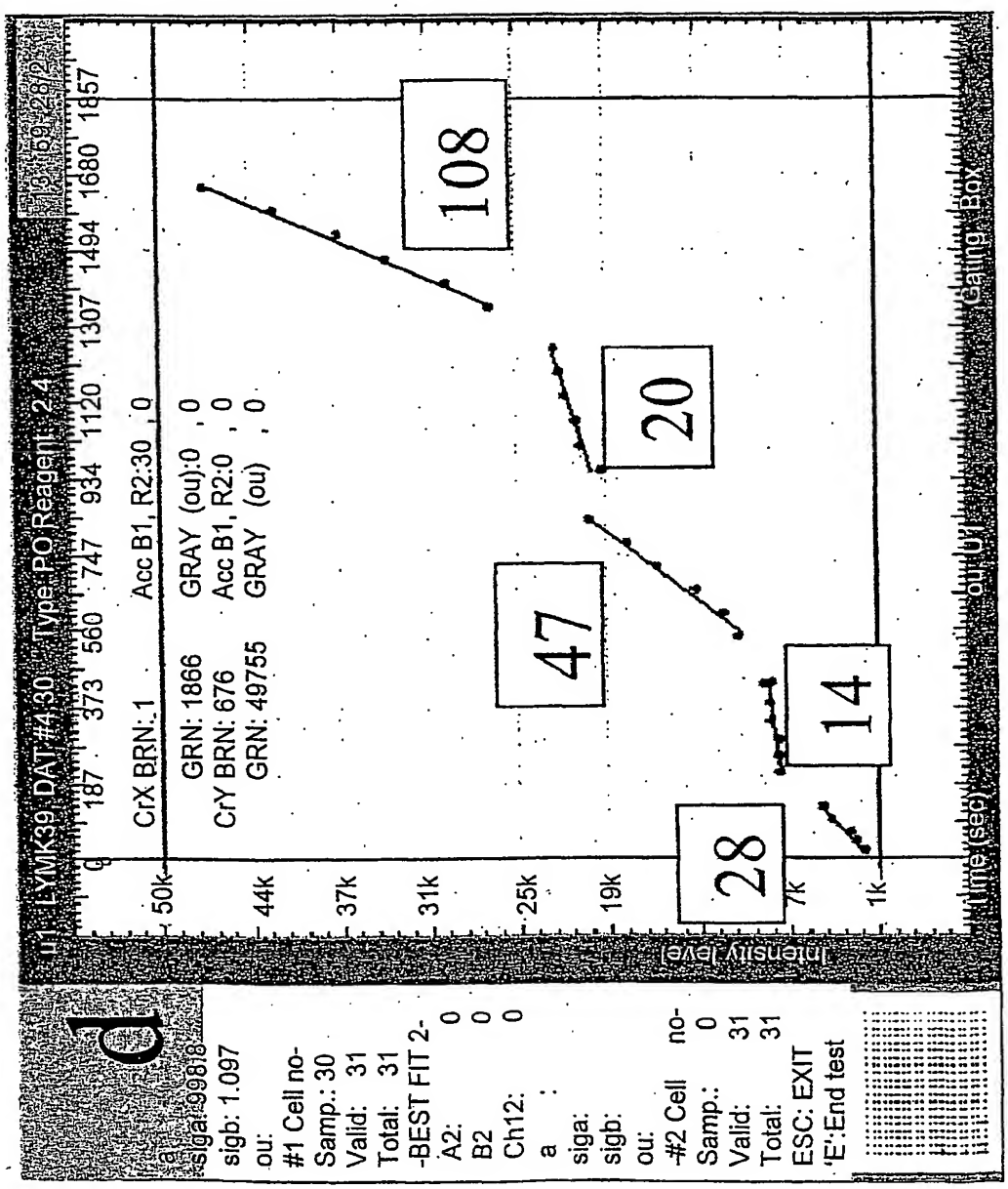

LYMK39 DAT #430 Type: PO Reagent: 24
CrX BRN: 1 Acc B1, R2:30 , 0
GRN: 1866 GRAY (ou):0 , 0
CrY BRN: 676 Acc B1, R2:0 , 0
GRN: 49755 GRAY (ou) , 0
Intensity leve
Time (sec)
Gating Box
108
47
20
28
14
sigb: 1.097
#1 Cell no-
Samp.: 30
Valid: 31
Total: 31
-BEST FIT 2-
A2: 0
B2 0
Ch12: 0
siga:
sigb:
#2 Cell no-
Samp.: 0
Valid: 31
Total: 31
ESC: EXIT
'E':End test

Fig. 3D

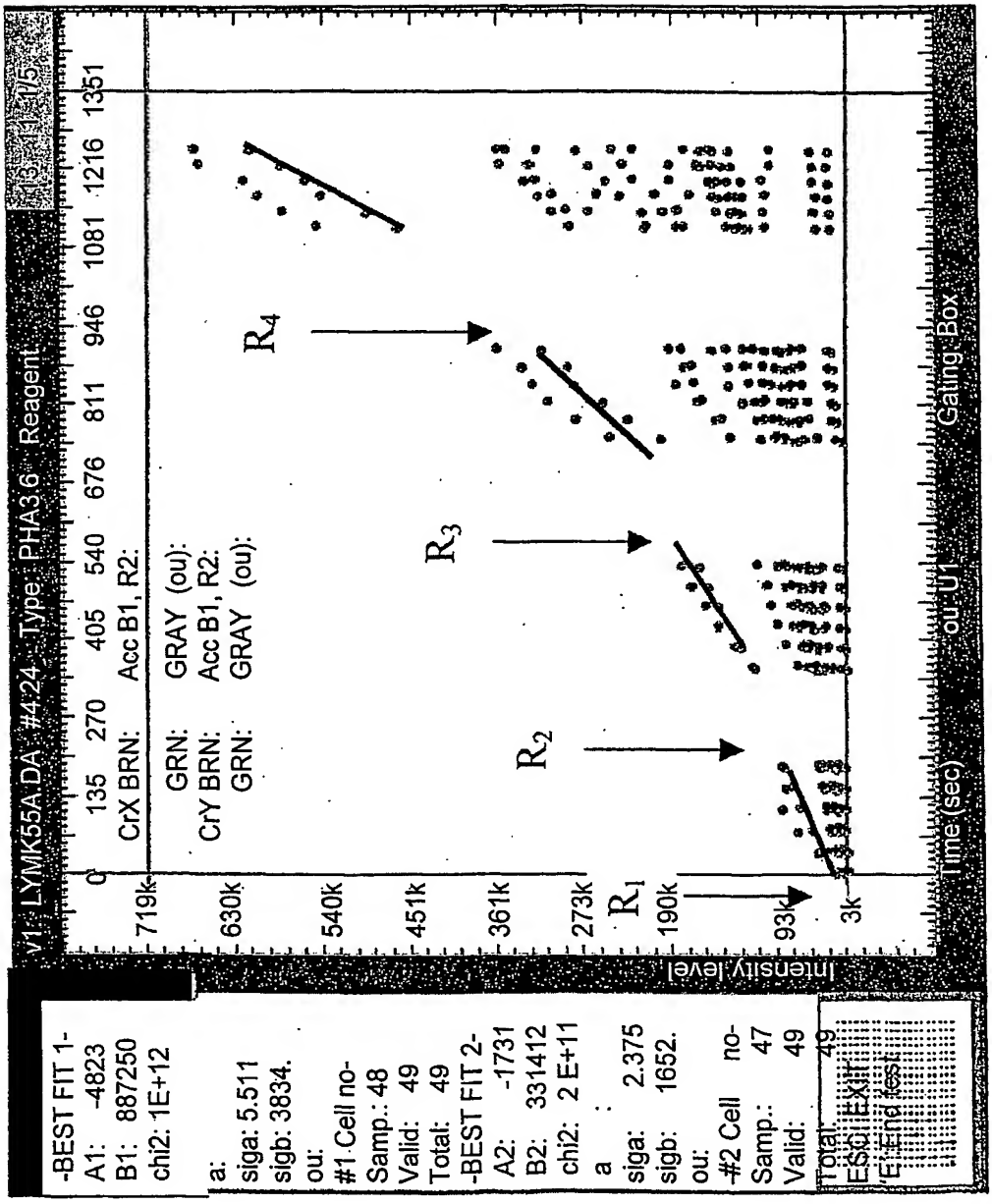

v1: LYMK55A.DA #4.24 Type: PHA3.6 Reagent
0 135 270 405 540 676 811 946 1081 1216 1351
CrX BRN: Acc B1, R2:
GRN: GRAY (ou):
CrY BRN: Acc B1, R2:
GRN: GRAY (ou):
719k
630k
540k
451k
361k
273k
190k
93k
3k
Intensity level
Time (sec)
Gating Box
R1
R2
R3
R4
-BEST FIT 1-
A1: -4823
B1: 887250
chi2: 1E+12
a:
siga: 5.511
sigb: 3834.
ou:
#1 Cell no-
Samp.: 48
Valid: 49
Total: 49
-BEST FIT 2-
A2: -1731
B2: 331412
chi2: 2 E+11
a :
siga: 2.375
sigb: 1652.
ou:
#2 Cell no-
Samp.: 47
Valid: 49
Total: 49
ESC: Exit
F: Find Test

Fig. 4

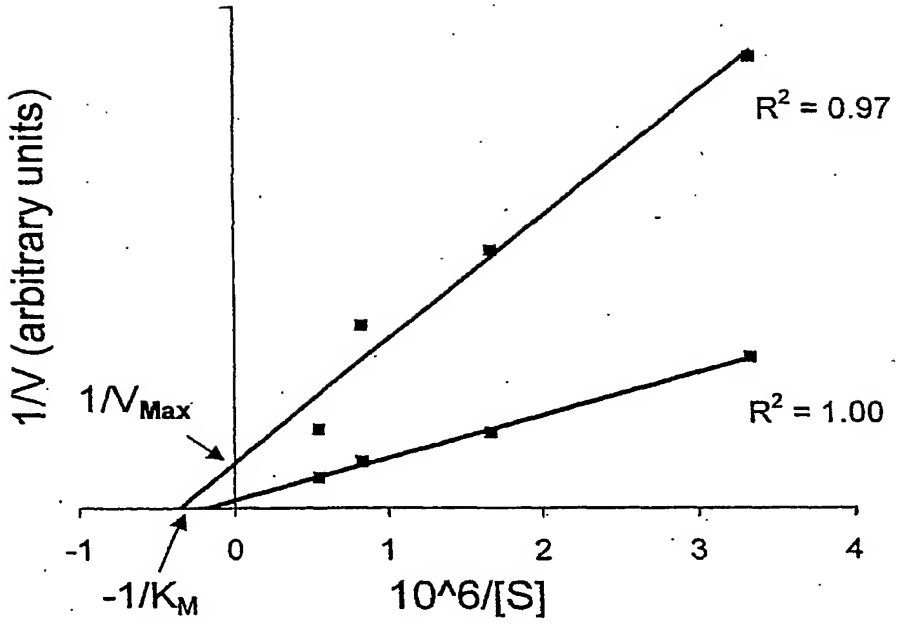


Fig. 5

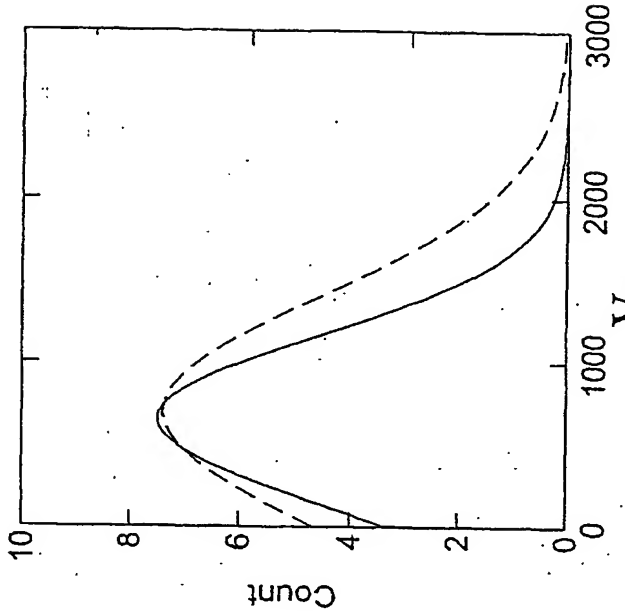

Count
10
8
6
4
2
0
0
1000
2000
3000
V_{MAX}

Fig. 6 B

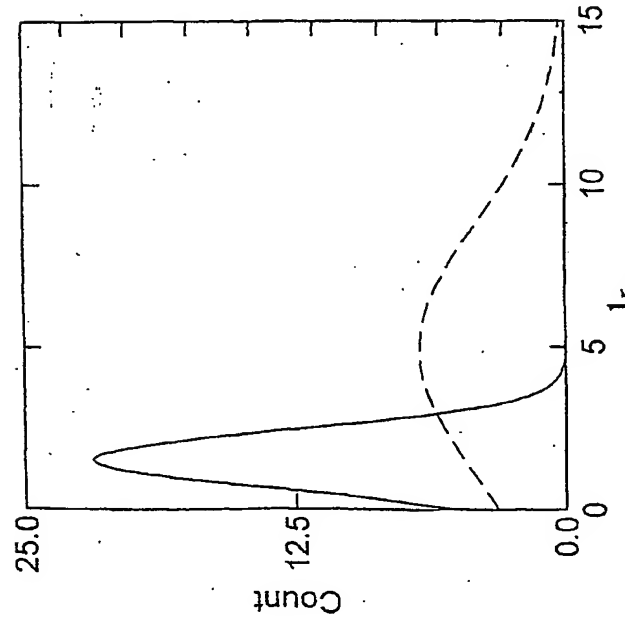

Count
25.0
12.5
0.0
0
5
10
15
K_M

Fig. 6A

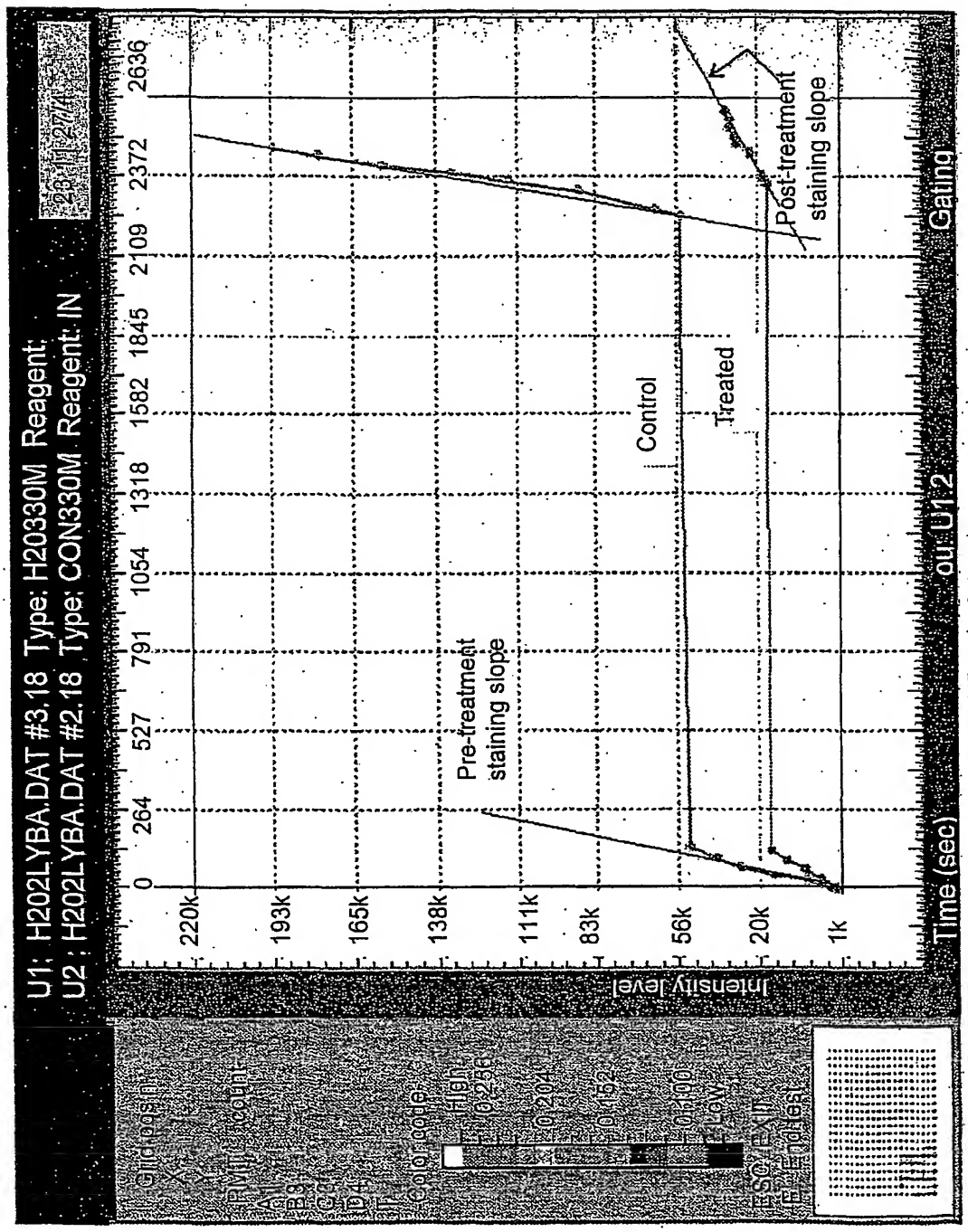

U1; H202LYBA.DAT #3.18 Type; H20330M Reagent;
U2 ; H202LYBA.DAT #2.18 Type; CON330M Reagent: IN
220k
193k
165k
138k
111k
83k
56k
20k
1k
0
264
527
791
1054
1318
1582
1845
2109
2372
2636
Intensity level
Time (sec)
Pre-treatment staining slope
Control
Treated
Post-treatment staining slope
Gating

Fig. 7

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 November 2001 (22.11.2001)

PCT

(10) International Publication Number
WO 01/88176 A3

(51) International Patent Classification[7]: C12Q 1/44, 1/37, 1/28, 1/54, 1/00

(21) International Application Number: PCT/IL01/00443

(22) International Filing Date: 17 May 2001 (17.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
136232 18 May 2000 (18.05.2000) IL

(71) Applicant *(for all designated States except US)*: **BAR-ILAN UNIVERSITY** [IL/IL]; 52900 Ramat-Gan (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SUNRAY, Merav** [IL/IL]; 7 Hahail Street, 43316 Raanana (IL). **ZURGIL, Naomi** [IL/IL]; 45 Borochov Street, 46240 Hertzlia (IL). **DEUTSCH, Mordechai** [IL/IL]; Moshav Olesh 73, 42855 Lev HaSharon (IL).

(74) Agents: **LUZZATTO, Kfir** et al.; Luzzatto & Luzzatto, P.O. Box 5352, 84152 Beer-Sheva (IL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

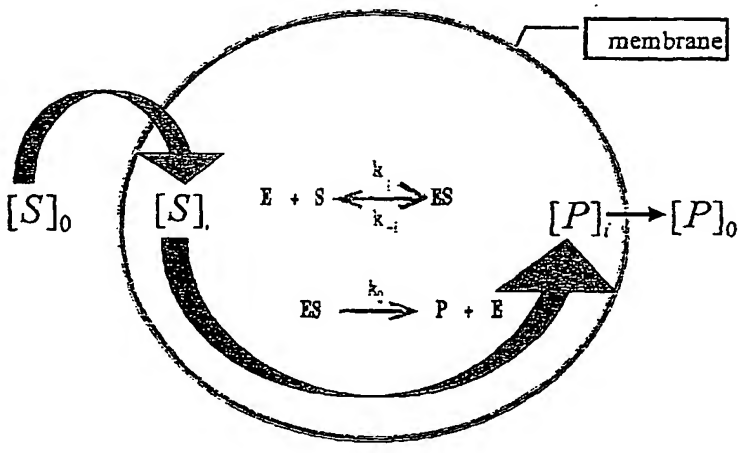
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
11 April 2002

[Continued on next page]

(54) Title: MEASUREMENTS OF ENZYMATIC ACTIVITY IN A SINGLE, INDIVIDUAL CELL IN POPULATION



(57) Abstract: A process for measuring enzymatic activity in an identified, isolated, intact, single, viable cell. Each of the viable cells is placed within individual identified locations on a carrier of a cytometer having means to measure enzymatic activity of a single viable cell placed in an identified location. The identified isolated cell is exposed to a substrate of an enzyme to be measured, and the rate of product formed or released following every exposure of the cell to same or different concentrations of the substrate is measured. The isolated cell may be exposed to a sequence of at least two different concentrations of the substrate, and for each exposure the rate of product formed or released is measured. Figure (1) is a model of intracellular conversion of a substrate to a product.

WO 01/88176 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL01/00443

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12Q 1/44, 1/37, 1/28, 1/54, 1/00
US CL : 435/19, 23, 24, 28, 14, 4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/19, 23, 24, 28, 14, 4, 968

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

west: enzymatic activity, viable cells, cytometer, substrate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,905,031 A (KUYLEN et al) 18 May 1999, see entire document.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 NOVEMBER 2001	Date of mailing of the international search report 27 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LOUISE LEARY Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998) *